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AUTHORITY

Phylis M. Rinehart, DCS for Info. Mgmt., U.S. Army Medical Research and Materiel Cmd. MCMR-RMI-S [70-1y] Ft. Detrick, MD

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13. ABSTRACT (Maximum 200 words)

Recombinant gp120 derived from HIV-1_{LAI} or HIV-1_{JR-FL} were expressed at high levels and purified. The purity of the recombinant proteins was demonstrated by the presence of a single band in SDS-PAGE analysis. Both recombinant gp120 were found to retain their binding to soluble CD4 and to cell surface expressed CD4, as determined by ELISA and immunofluorescence staining, respectively. The reciprocal anti-gp120 antibody titers induced by unconjugated recombinant gp120 were in the range of 250,000 to 500,000 in guinea pigs, whereas much lower antibody titers were observed in BALB/C and C57BL mice (5,000 to 6,000). Recombinant gp120 conjugated to tetanus toxoid in the presence of glutaraldehyde induced significantly lower antibody responses in BALB/C and C57BL mice, as compared to unconjugated proteins. Although the glutaraldehyde-conjugated gp120 retained its binding to CD4, it appears that the conjugation process reduced its immunogenicity. Therefore, we have conjugated the recombinant gp120 proteins to KLH, characterized the gp120-KLH conjugates and initiated immunogenicity studies of the conjugates in rodents.

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INTRODUCTION

It has been repeatedly demonstrated by a number of investigators that immunization of both humans and various animal models with antigenic products based on the envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1) results, at best, in the generation of low neutralizing anti-HIV-1 antibody titers. These results clearly suggest that present HIV-1 envelope vaccine approaches may not be effective. In addition, the transient nature of the antibody responses and the predominance of isolate-specific neutralizing activity have also been discouraging to efforts aimed at developing an effective HIV-1 vaccine (Cohen 1993). Similar results confounded early investigators utilizing polysaccharide (Ps) and carbohydrate antigens as vaccine candidates for bacterial and parasite pathogens. These studies demonstrated weak immunogenicity, especially in infants who were most susceptible to infections caused by these pathogens. This has promoted a number of conjugate vaccine studies in which the Ps antigen with low immunogenicity was covalently coupled to a highly immunogenic protein antigen (i.e., tetanus toxoid, the outer membrane protein complex of *Neisseria meningitidis* (OMPC), diphtheria toxoid, among others) (reviewed in Vella and Ellis 1992). These early Ps conjugate-based vaccines were designed to activate a carrier-specific T-lymphocyte response, which in turn drives B-lymphocytes toward the production of antibodies specific for the non-immunogenic target antigen or hapten; this is commonly referred to as the well-characterized hapten-carrier phenomenon. The conjugate-based vaccines have met with mixed success in experimental animal models. In some instances, conjugated antigens have proven highly immunogenic in promoting the desired enhancement of anti-pathogen antibody responses, whereas, in other studies the addition of a carrier antigen significantly suppressed the desired antibody response (Herzenberg et al., 1980; Schutze et al., 1985).

Over the past several years, conjugate vaccines have met with much greater success and a number of these vaccines have received United States Food and Drug Administration (FDA) approval for use in adults and infants (i.e., *Haemophilus influenzae* type B, group B *Streptococcus* type III, *Pneumococcus*, among others). Thus, it is the overall objective of this research project to assess the potential of an HIV-1 envelope glycoprotein (gp120) conjugate-based vaccine to induce high, long-lasting anti-HIV-1 envelope antibody titers with group specific neutralizing activity against primary and laboratory isolates of HIV-1.

RESULTS

Specific Aim #1: *Produce and characterize 100 mg of HIV-1 gp120 derived from the LAI and JR-FL strains for use in conjugation and immunization experiments.*

Development and culture of cell lines expressing recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120:

We have previously described the generation of recombinant Chinese Hamster Ovary (CHO) cell lines that express high levels of either the laboratory-adapted T-cell tropic HIV-1_{LAI} gp120 protein or the primary macrophage tropic isolate HIV-1_{JR-FL} gp120 protein. To briefly summarize, the nucleotide sequences encoding either gp120 protein were inserted into the optimized mammalian expression vector PPI3-gp120, where gp120 expression is under the control of the cytomegalovirus MEI promoter and enhancer regions. The vector also encodes the gene for dihydrofolate reductase, which imparts resistance to methotrexate (MTX). PPI3-gp120 was transfected into DXB-11 dhfr-CHO cells. Positive clones were selected for growth in nucleoside-free media, amplified by step-wise increases in the concentrations of MTX, and adapted for growth in reduced serum media.

For large-scale production, fifty roller bottles were seeded with clones secreting gp120 from either virus strain and grown to confluency. Supernatant collections were performed twice weekly in ExCell 301 media (JRH Biosciences) containing 2% fetal bovine serum (FBS, Gibco-BRL). Greater than 50L of conditioned media was collected, sterile filtered, and stored frozen at -95 °C prior to purification. Each gp120 protein was expressed at 5-10 mg/L for a total of 250-500 mg prior to downstream processing.

Purification of recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120:

We have incorporated a third column, namely gel filtration, into the purification process described previously. The optimized process is performed entirely at neutral pH in the absence of chaotropic or denaturing agents and has proven equally effective in purifying recombinant gp120 from either the LAI or JR-FL strain of HIV-1. Briefly, clarified roller bottle supernatant is passed over a *Galanthus nivalis* (GNA) column. The GNA lectin recognizes glycans containing terminal mannose residues, which are found extensively on native gp120 (Gilljam, 1993). The GNA column is prepared at Progenics by coupling GNA lectin (Boehringer Mannheim) to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. After extensive column washes, glycoproteins are competitively eluted with 0.5M methyl-α-D-mannopyranoside in PBS. The GNA eluate is dialyzed against 20mM imidazole buffer at pH 7.1, loaded onto a Q High Performance anion exchange column (Pharmacia) equilibrated in the same buffer, and eluted with step-wise increases in sodium chloride concentration. The post-Q material is concentrated and loaded onto a Superdex 200 gel filtration column equilibrated in PBS. The entire purification process is carried out at 4 °C.

Purity analysis of HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 by SDS-PAGE:

Purified gp120 was reduced with dithiothreitol, loaded onto a pre-cast 4-15% Ready Gel (Bio-Rad Laboratories), run on SDS-PAGE, and visualized with coomassie blue. The destained gel was scanned using a laser densitometer (Molecular Dynamics). As shown in Figure 1, the purified gp120 proteins each ran as a single diffuse band with apparent molecular weights near 120kd, as expected for proteins bearing native glycosylation patterns. In comparison with HIV-1_{LAI} gp120, HIV-1_{JR-FL} gp120 contains 13 fewer amino acids and one less site of potential N-linked glycosylation. As expected from these differences in primary sequence, recombinant HIV-1_{JR-FL} gp120 runs with a measurably increased mobility on SDS-PAGE. The purity of each preparation was estimated as >95% using ImageQuant software (Molecular Dynamics). In total, the gel results suggest that the production process yields gp120 preparations which are highly purified, free of degradation products, and possessed of native glycosylation patterns.

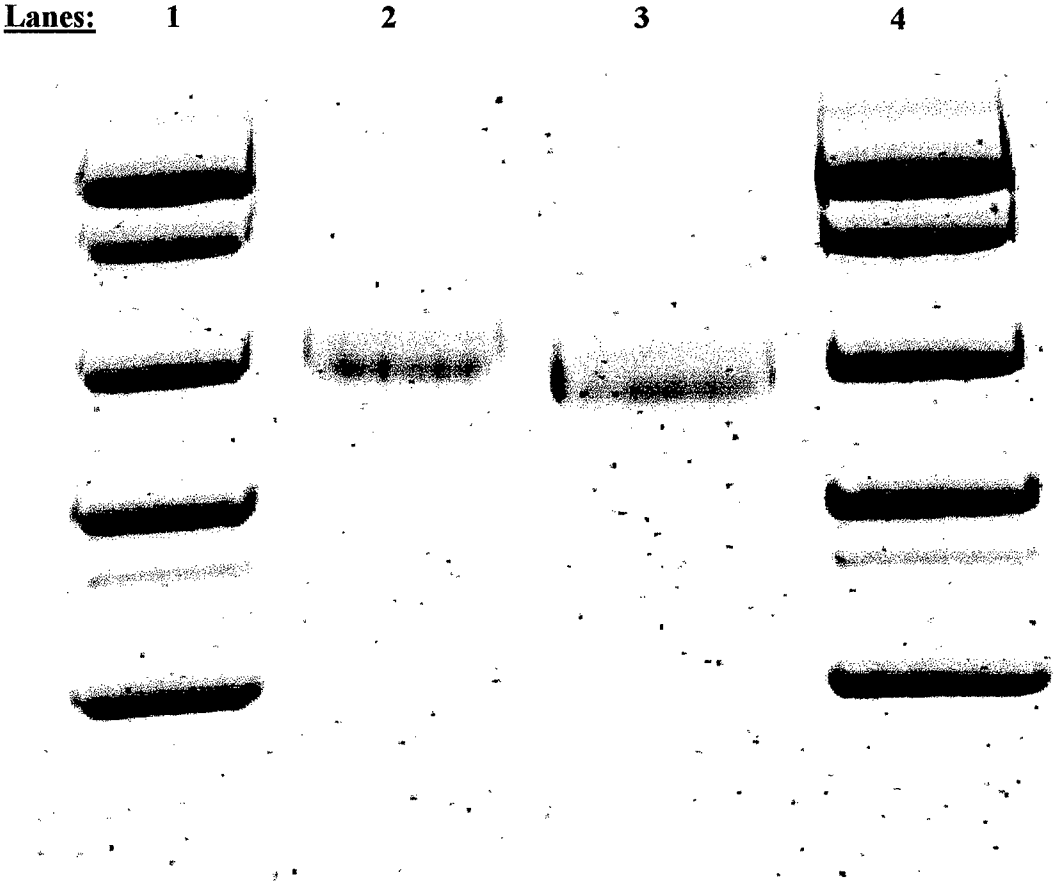

Figure 1. Scanned image of an SDS-PAGE gel comparing purified, recombinant HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120. A 4-15% Ready-Gel (Bio-Rad Laboratories) was run under reducing conditions, stained with coomassie blue, and scanned on a laser densitometer (Molecular Dynamics).

Lanes 1 & 4: Pharmacia high molecular weight markers (212kd, 170kd, 116kd, 76kd, 53kd)
Lane 2: HIV-1_{LAI} gp120, 10 μg
Lane 3: HIV-1_{JR-FL} gp120, 10 μg

Analysis of gp120 binding to CD4-based proteins:

The purified recombinant gp120 proteins from HIV-1_{LAI} and HIV-1_{JR-FL} were tested for their abilities to bind soluble CD4 (sCD4) and CD4-IgG2 in an ELISA format. Both CD4-based proteins were prepared by Progenics. CD4-IgG2 is a novel heterotetramer comprising two chains of a CD4-human IgG2 heavy chain fusion protein and two chains of a CD4-human κ fusion protein; its expression, characterization, and anti-viral properties have been recently described (Allaway et al., 1995; Trkola et al., 1995). Briefly, gp120 was captured onto an ELISA plate (Dynatech Laboratories, Inc.) at 5 ng/well using a polyclonal antibody to the conserved C terminus of gp120 (International Enzymes, Inc.). The plate was blocked by incubating with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 hr. Purified sCD4 or CD4-IgG2 was then added in a range of concentrations (e.g., 0.1-100 nM) to the immobilized gp120 and incubated for 1 hr at 37 °C. Following wash steps, the amount of bound sCD4 was determined by incubation with the mouse anti-CD4 monoclonal antibody OKT4 (Ortho Diagnostic Systems, Inc.), followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Inc.) and then with *o*-phenylenediamine (OPD) substrate. Bound CD4-IgG2 was detected by incubation with HRP-labeled affinity purified goat antibody specific for human Ig Fc, followed by OPD substrate. Absorbance readings were measured at 492 nm using an automated ELISA plate reader. All wash and incubation steps employed PTB buffer (0.5% Tween 20, 1% FBS, and 0.1% BSA in PBS).

The binding data are summarized in Table 1 and are expressed as EC_{50} values, the concentration of CD4-based protein giving half-maximal binding. The EC_{50} value is a reasonable approximation of the thermodynamic dissociation constant (Orloff et al., 1993; Moore et al., 1991). The recombinant gp120 molecules from both the laboratory-adapted strain and the primary isolate bound to CD4-based proteins with nanomolar affinity. These results compare favorably with binding data obtained using gp120 extracted from virions (Moore et al., 1990) and suggest that the purified recombinant proteins are conformationally intact.

	EC_{50} (nM)	
CD4-based Protein	***HIV-1_{LAI}***	***HIV-1_{JR-FL}***
sCD4	1.93	2.22
CD4-IgG2	1.25	0.64

Table 1. Comparison of HIV-1_{LAI} gp120 and HIV-1_{JR-FL} gp120 binding to CD4-based proteins in an ELISA format.

Analysis of recombinant HIV-1_{LAI} and HIV-1_{JR-FL} gp120 binding to cell surface CD4:

To characterize the purified gp120's further, their binding to cell surface CD4 was analyzed by flow cytometry. Each gp120 was incubated at 5μg/ml for 2 hours at 37°C in FACS buffer (PBS +

2%BSA + 0.1% azide) with a CHO cell line engineered at Progenics to express high levels of full length human CD4. The cells were then washed and incubated with a sheep antibody to the conserved C-terminus of gp120. After another wash, the cells were incubated with FITC-conjugated rabbit anti-sheep Ig, washed and fluorescence determined using a Becton Dickinson FacScan.

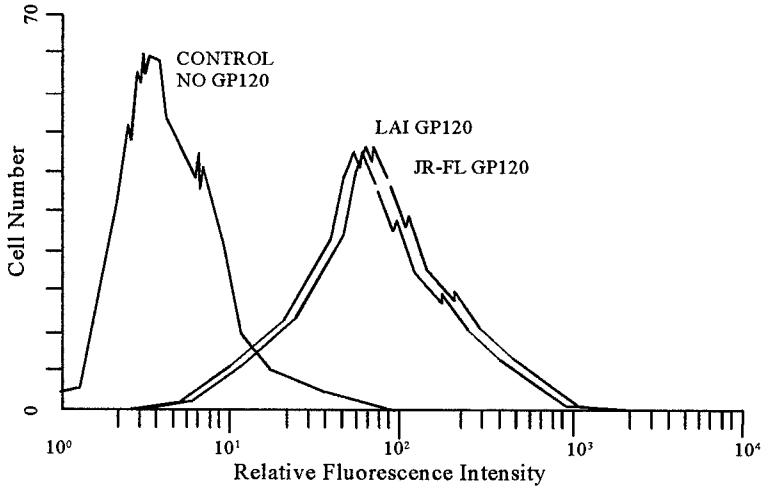

Figure 2. Flow cytometric analysis of LAI and JR-FL gp120 binding to $CD4^+$ CHO cells.

The results shown in Figure 2 indicate that both gp120's bind well to the CD4-bearing cells, further confirming that these molecules are in their *native conformation* with intact CD4-binding sites. No binding was seen using control, CD4-negative cells (not shown).

Analytical gel filtration analysis of purified HIV-1_{LAI} and HIV-1_{JR-FL} gp120 proteins:

The aggregation state of the purified gp120 proteins was determined by analytical gel filtration on a TSK G3000SW_{XL} column (TosoHaas) run in 100mM sodium phosphate, 100 mM sodium sulfate buffer, pH 6.7. The column elution profiles are shown in Figure 3. Both HIV-1_{LAI} and HIV-1_{JR-FL} gp120 proteins were >95% monomeric when purified using the optimized three-column method. As also shown in Figure 3, the HIV-1_{JR-FL} gp120 protein was approximately 70% monomeric prior to gel filtration. Similar results were obtained for HIV-1_{LAI} gp120 purified using the two-column method (data not shown). The remaining material consisted primarily of high molecular weight species which have been tentatively identified as disulfide-linked gp120 aggregates that bind sCD4 poorly in ELISA.

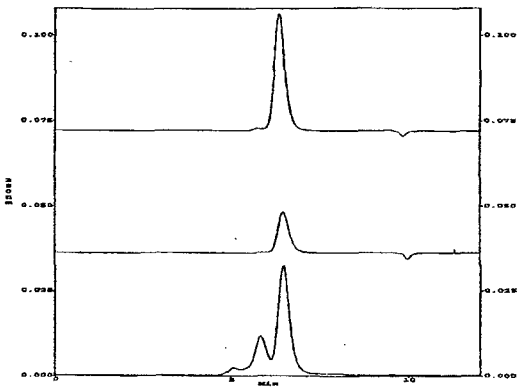

Figure 3. Analytical gel filtration analysis of purified gp120. Top: HIV-$_{LAI}$ gp120 purified via the 3-column process; middle: HIV-1_{JR-FL} gp120 purified via the 3-column process; bottom: HIV-1_{JR-FL} purified via a 2-column process.

Analytical gel filtration analysis of the gp120-CD4 binding interaction:

The ELISA described above is useful in gauging the strength of the gp120-CD4 interaction. However, denatured gp120 molecules would not be detected. To assess whether the majority of gp120 molecules present in our preparations were capable of binding to CD4, an analytical gel filtration binding assay was developed. In this assay, HIV-1_{LAI} gp120 was incubated with a slight molar excess of sCD4 for 1 hr at 4°C in PBS and then injected onto a TSK G3000SW$_{XL}$ column (TosoHaas) equilibrated in PBS. As indicated in Figure 4, the addition of sCD4 caused the gp120 peak at 6.2 min (Figure 4, middle profile) to disappear and a new peak to appear at 6.0 min (Figure 4, lower profile). The new peak is presumably the gp120-sCD4 molecular complex. In the profile for the mixture (Figure 4, lower profile), the absence of split peaks or even a defined shoulder at 6.2 min suggests that the gp120 preparation is essentially fully reactive. The high specific activity of the preparation can be attributed to the fidelity of the mammalian cell expression system and the mild conditions employed during purification.

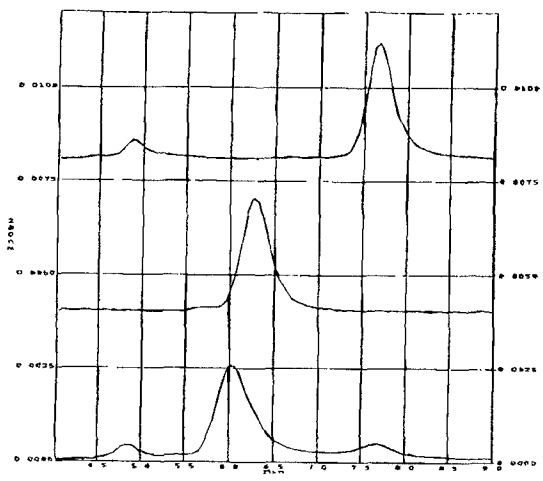

Figure 4. Analytical gel filtration analysis of gp120-sCD4 binding. Samples were injected onto a G3000SW$_{XL}$ column (Toso-Haas) run at 1ml/min in PBS buffer. Top: 5 μg sCD4; middle: 10μg HIV-1_{LAI} gp120; bottom: 5 μg sCD4 + 10 μg HIV-1_{LAI} gp120 pre-incubated for 1 hr at 4 °C.

Immunogenicity of unconjugated HIV-1_{LAI} and HIV-1_{JR-FL} gp120's in two adjuvants:

The HIV gp120's prepared as described above were initially tested for immunogenicity in the native form, prior to conjugation. Guinea pigs (3 animals per group) were inoculated 5 times with 100μg injections of either gp120 strain in alum or Ribi Detox adjuvants. The immunization schedule included subcutaneous and intraperitoneal administrations. Bleeds were performed prior to and at intervals after the first injection. The humoral response was determined by ELISA. Briefly, gp120 of the same strain used for immunization was immobilized on an ELISA plate. Following blocking and washing steps, dilutions of serum from 1:8000 to 1:512,000 in PBS/Tween were added. The level of bound antibody remaining after washing was determined using peroxidase-labeled goat anti-guinea pig Ig. The greatest serum dilution giving a signal 2x that obtained with pre-immune serum was defined as the titer. As shown in Figure 5, the reciprocal serum titers increased following sequential immunizations to a maximum of 250,000 to 500,000. Similar results were obtained using LAI and JR-FL gp120's. Moreover, while early titers were higher with the Ribi adjuvant, the maximum titers achieved using both adjuvants were similar.

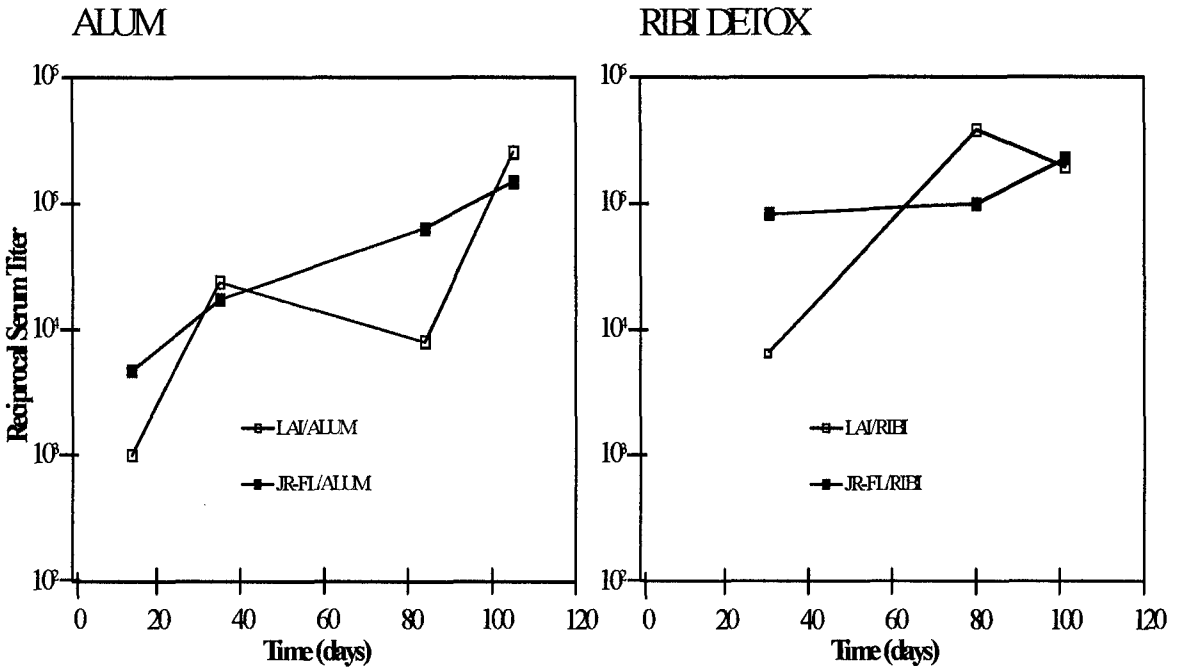

Figure 5. Comparison of anti-gp120 titers exhibited in guinea pigs immunized with LAI and JR-FL gp120's using alum or Ribi-Detox adjuvants

Next we examined the sera from these immunized guinea pigs to determine the level of antibodies to the CD4-binding site of gp120, capable of blocking the gp120-CD4 interaction. Such antibodies would be expected to neutralize a broader range of HIV-1 strains than antibodies to other epitopes such as the V3 loop. Briefly, immune sera from the animals were dialyzed and the immunoglobulin fraction prepared by Protein A affinity chromatography. ELISA plates were coated with sCD4, then incubated with biotinylated gp120 which had been pre-incubated (2 hours at room temperature) with dilutions of the immunoglobulin preparations. The amount of gp120 bound was determined using peroxidase-conjugated avidin and compared with the maximum bound in the

absence of antibodies. The antibody dilution which inhibited binding by 50% was defined as the gp120-CD4 blocking titer. The results are shown in Table 2, converted to the equivalent serum titer.

Immunizing gp120	*Adjuvant*	*Blocking titer against LAI gp120*	*Blocking titer against JR-FL gp120*
LAI	Alum	381	345
LAI	Ribi Detox	2233	725
JR-FL	Alum	73	211
JR-FL	Ribi Detox	52	427

Table 2. gp120-CD4 blocking titers generated in guinea pigs immunized with HIV-1_{LAI} and HIV-1_{JR-FL} gp120 proteins in two adjuvants.

All sera contained antibodies capable of blocking CD4 binding, in each case being more potent in the assay using homologous gp120. Higher blocking titers were obtained in most cases using Ribi Detox as an adjuvant compared with alum, presumably because the latter denatures the immunogen, thereby inhibiting the generation of antibodies to conformational epitopes such as the CD4 binding site on gp120.

***Specific Aim #2**: Conjugate tetanus toxoid and diphtheria toxoid carriers to the gp120 molecules using a variety of established chemistries. Evaluate both the integrity of gp120 after conjugation and the immunogenicity of the HIV-1 subunit conjugates in mice.*

Preparation and Immunogenicity of HIV-1_{LAI} and HIV-1_{JR-FL} gp120 conjugated to tetanus toxoid:

The fixative glutaraldehyde is a non-specific, mildly denaturing agent which has proven as an efficient reagent for protein to protein coupling (Harlow and Lane, 1988). Briefly, tetanus toxoid (TT) and HIV-1 gp120 are mixed at a 2.5:1 ratio in a solution of glutaraldehyde in sodium phosphate, pH 6.0, for 2 hours at room temperature. Excess glutaraldehyde is removed by dialysis against borate buffered saline. The binding of the conjugates to CD4, assessed as discussed below, showed that the conjugates retain their binding to CD4 (data not shown).

The gp120 or gp120-TT were precipitated in aluminum hydroxide and used to vaccinate groups of BALB/C and C57BL mice. Three immunizations of 5.0 μg HIV-1_{LAI} or HIV-1_{JR-FL} gp120 each were given intramuscularly on a monthly basis and the antibody titers were determined by ELISA essentially as described above. The results given in Table 3 suggest that conjugation of HIV-1_{LAI} gp120 to TT elicited lower anti-gp120 antibody titers as compared to those induced by unconjugated gp120. The reason for this is unclear at this time. Similar results were obtained with HIV-1_{JR-FL} and are not shown.

Mouse Inbred Strain	No. of Mice	Immunogen	No. of Immunizations	End-Point Antibody Titers
C57BL/6	5	gp120/Alum[a]	3[b]	5,870[c] (2,000-11,600)[d]
	3	gp120-TT/Alum	3	1,433 (750-2,150)
BALB/C	5	gp120/Alum	3	6,160 (3,200-10,400)
	4	gp120-TT/Alum	3	2,432 (163-6,800)

[a] 5.0 μg of gp120 were given per injection.
[b] Immunizations were given monthly.
[c] Mean antibody titers.
[d] Range of antibody titers

Table 3. Antibody responses in BALB/C and C57BL mice immunized with unconjugated gp120 or gp120 conjugated to tetanus toxoid (gp120-TT) administered in alum.

Studies have indicated that priming of the immune response to a specific carrier protein suppresses the ability of the individual to respond to the epitope associated with the haptenic portion following a subsequent immunization with a hapten-carrier protein (Herzenberg et al., 1980; Schutze et al., 1985). However, these studies were performed in inbred strains of mice. Other studies have indicated that priming with the carrier protein can either enhance or suppress the immune response to the Ps moiety following a subsequent vaccination with Ps-carrier protein conjugate vaccines (Schneerson et al., 1984; Vella and Ellis, 1991; Granoff et al., 1993; Peeters et al., 1991; Lieberman et al., 1993). Overcoming carrier protein-induced immunosuppression has been observed by utilizing alternative carrier proteins, and by modifying the dose administered in subsequent vaccinations (Gaur et al., 1990). In addition, the age of the individual at the time of immunization also represents a parameter associated with carrier protein priming and suppression (Granoff et al., 1993; Lieberman et al., 1993). Interestingly, a number of investigators have noted that carrier suppression can be bypassed in humans by modifying the carrier protein (Marcinkiewicx et al., 1992) or by performing appropriate dosing studies (Di John et al., 1989). Vaccinations with higher doses have been shown to successfully eliminate carrier suppression and significantly boost and stabilize the desired anti-pathogen responses (Di John et al., 1989). Specifically with Hib conjugate vaccines, reports have indicated that pre-existing immunity to the carrier protein can enhance (Granoff et al., 1993; Burmington et al., 1993), or suppress (Lieberman et al., 1993) the immune responses to Hib PRP following subsequent vaccination. In addition, whether the antibodies to the carrier protein were actively induced or passively acquired from the mother during pregnancy has also been reported to affect the ability of infants to respond to subsequent Hib conjugate vaccination (Barrington et al., 1994). Studies have suggested that high titers of maternally-derived passive antibodies against *Salmonella* flagellin inhibited the ability of the neonates to respond to a *Salmonella* vaccine, while neonates lacking antibodies respond as well as adults. Specific immunosuppressive effects of transplacentally acquired antibodies to diphtheria toxin and poliomyelitis have also been reported in

infants. Whether this suppression with glycoconjugate vaccines results from clonal competition by carrier-reactive B-lymphocytes (Schutze et al., 1989) or by the induction of suppressor T-lymphocyte activities (Herzenberg et al., 1980) remains to be determined. Thus, the question related to the effects of pre-existing antibodies to the carrier moiety on the ability to respond to a subsequent vaccination with a conjugate formulation remains unclear and is problematic for developing an HIV-1 gp120 conjugate vaccine.

Because of the relatively low anti-gp120 antibodies titers in mice vaccinated with gp120-TT described above and the possibility of immunosuppression induced by pre-existing anti-TT antibodies in humans, we have selected to conjugate gp120 to KLH for future immunogenicity studies.

Use of keyhole limpet hemocyanin (KLH) as a protein carrier to improve the immunogenicity of gp120:

As discussed above, the gp120-TT conjugates exhibited poor immunogenicity when injected into mice and may be subject to carrier-induced immunosuppression in TT-exposed humans. Therefore, an alternative approach was used, whereby the gp120s were conjugated with KLH. As a carrier for conjugate vaccines, this protein has the advantage over tetanus toxoid in that individuals are unlikely to have a pre-existing immune response to the carrier alone, which could limit the efficacy of conjugate vaccination. Several studies have found that KLH can enhance the antibody response to antigens, often more effectively than other carrier proteins, possibly because its size and antigenic complexity aid antigen processing and recruitment of T cell help (Helling et al., 1994; Longenecker et al., 1994). Clinical studies using various conjugates incorporating KLH as cancer vaccine candidates have demonstrated improved humoral immune responses and clinical benefits following treatment with these conjugates (Longenecker et al., 1994; Helling et al., 1995).

Our studies employed a KLH preparation (PerImmune, Inc.) which has been administered to human subjects under IND's from multiple companies, including Progenics. In SDS-PAGE, the protein migrates with a major band at approximately 275kd. In nondenaturing assays such as sucrose gradient centrifugation or gel filtration, the protein migrates as an 8.6×10^6 dalton noncovalent multimer.

Preparation of gp120-KLH conjugates:

We examined several conjugation chemistries which target various functional groups of gp120, including carbohydrate moieties, primary amines, and carboxyl groups. To minimize modification of the gp120 protein, two-step procedures were followed whenever possible whereby KLH was the protein modified more extensively. The conjugates were analyzed for CD4-binding activity in a sandwich ELISA using anti-KLH antibodies to capture the conjugate. This ELISA is specific for gp120-KLH conjugates in that both KLH and gp120 epitopes must be present on the same molecule for a positive reaction to occur. In addition, the conjugates were analyzed by SDS-PAGE to determine the extent of crosslinking. HIV-1_{LAI} gp120 was used in all of the conjugation studies described below.

N-hydroxysuccinimidyl-2-3-dibromopropionate (SDBP) is a heterobifunctional crosslinking agent containing two amine-reactive moieties which vary in temperature sensitivity. That is, the reactivity of the alkylhalide group is nearly negligible at 4 °C but greatly improved at 25 °C, while the succinimide moiety is reactive throughout this temperature range. SDBP yields conjugates linked

by a stable aziridine group and a 2-carbon spacer element. SDBP was used to crosslink KLH and gp120 in the following two-step procedure. In the first step, carried out at 4 °C, 2-4 mg of SDBP was dissolved in 200 µl of DMSO, buffered with 40µl of PBS, and then added to 2mg KLH in 400 µl PBS. After a 2 hr reaction favoring the *N*-hydroxysuccinimidyl moiety of the crosslinker, KLH was dialyzed against PBS. In the second step, an equal mass of gp120 in PBS was added, and the mixture was warmed to ambient temperature to promote the reaction between alkylbromide-activated KLH and gp120. Following a 16 hour incubation, the conjugate was further dialyzed against PBS.

In addition to the expected Schiff base formation, glutaraldehyde-mediated crosslinking is thought to proceed by mechanisms involving unsaturated glutaraldehyde polymers (Wong, 1991). These Michael-type addition products are stable in the absence of reducing agents such as sodium borohydride. Both one- and two-step glutaraldehyde coupling procedures were examined (Hermanson, 1996). In the more successful two-step procedure, KLH was reacted for 1 hr with 0.075% glutaraldehyde in 50mM phosphate buffer at pH 7.5 and then dialyzed against PBS at 4 °C. At this time, gp120 in PBS was added at an equal mass ratio and reacted for 2hr at 4 °C. Tris was then added to 20mM to block any remaining active sites and the reaction was continued for an additional hour at 4 °C, after which the conjugate was dialyzed against PBS.

Analysis of gp120-KLH conjugates for CD4 binding activity:

Conjugates were tested in a sandwich ELISA specific for gp120-KLH conjugates possessing a functional CD4-binding site. In the assay, Probind 96-well microtiter plates (Falcon) are coated with polyclonal rabbit anti-KLH antibodies (prepared by the Progenics Oncology program) at 100 ng/well. The plates are washed with PBS/0.02% Tween 20 (PBST) and blocked with PBS containing 5% BSA. To complete the assay, the following reagents are added with intermediate PBST washes: (1) gp120-KLH conjugate, typically at 50ng/well, (2) 2-150 nM sCD4, (3) the murine anti-CD4 monoclonal antibody OKT4 (Ortho Diagnostics), (4) HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry), and (5) OPD substrate. Signals are read at 492 nm on an ELISA plate reader, and the data are fit to a Langmuir isotherm using SlideWrite Plus. Background readings are routinely obtained in wells containing gp120, KLH, or mixtures of unconjugated gp120 and KLH.

As indicated in Figure 6, appreciable CD4-binding activities were observed for conjugates prepared with SDBP and with glutaraldehyde. These conjugates were prepared using a 1:1 gp120:KLH mass ratio. The estimated equilibrium constants ranged from 24 nM to 66 nM and are approximately 10-fold higher than those observed for monomeric gp120 in similar assay systems. As neither conjugation procedure is site-directed, the CD4-binding site is expected to be variably accessible in the conjugated molecules, reducing the average affinity of the interaction. We emphasize that this analysis does not determine true thermodynamic dissociation constants because (1) the equilibrium concentrations of unbound sCD4 are not measured and (2) the equilibrium is disturbed by several wash steps prior to detection. Both factors would tend to underestimate the strength of the interaction.

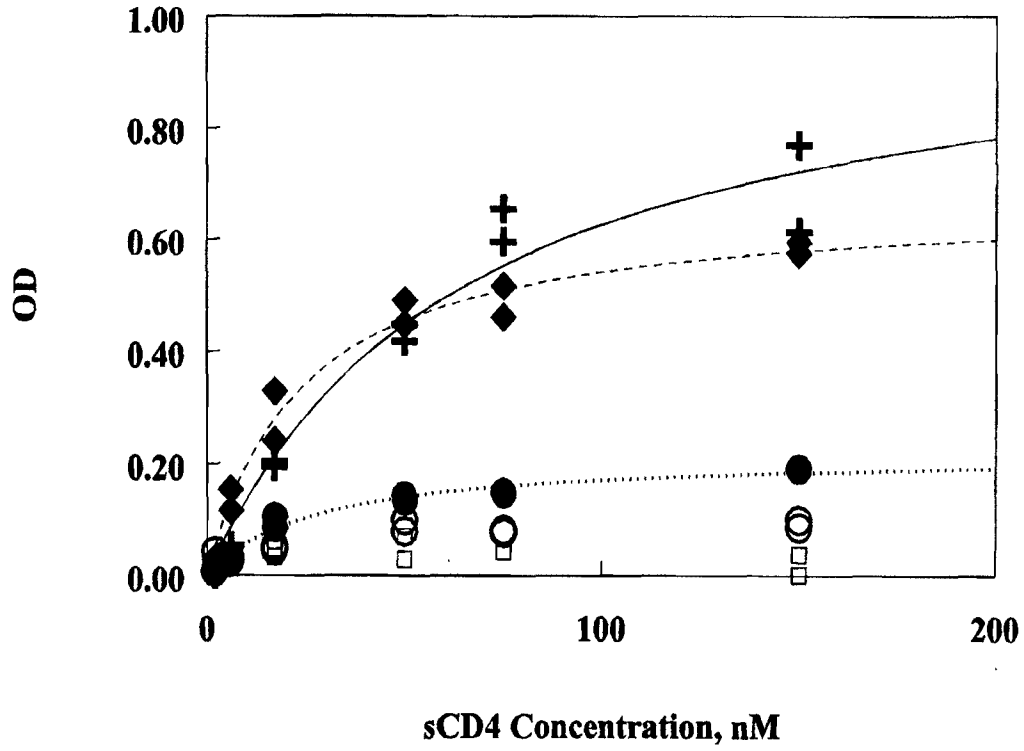

Figure 6. Binding of gp120-KLH conjugates to sCD4 in a microplate assay. Conjugates were prepared using 3mg/ml SDBP (+); 6mg/ml SDBP (□); 0.075% glutaraldehyde in a two-step procedure (♦); 0.25% glutaraldehyde in a two-step procedure (•); and 0.25% glutaraldehyde in a one-step procedure (O). Conjugates were prepared using HIV-1_{LAI} gp120 in a 1:1 mass ratio with KLH. The data were fit to a Langmuir isotherm using nonlinear regression.

Conjugate binding properties were critically affected by crosslinking conditions. The highest CD4 binding activity was observed for conjugates prepared using 3 mg/ml SDBP; conjugates prepared using 6 mg/ml SDBP were inactive. Similarly, the two-step glutaraldehyde procedure was more effective at 0.075% glutaraldehyde than at higher crosslinker concentrations. A one-step glutaraldehyde cross-linking procedure was ineffective at 0.25% glutaraldehyde. One possibility is that at higher concentrations of crosslinker, the gp120 molecule becomes conjugated through multiple covalent bonds that distort the CD4 binding site.

Additional chemistries failed to yield conjugates which were reactive in the above ELISA. Since gp120 glycans are reportedly dispensable for CD4-binding (Fenouillet et al. 1989, 1996), conjugates were initially prepared using reductive amination under a variety of test conditions. In the basic procedure, vicinal diols on gp120 glycans were oxidized to aldehyde groups using sodium *m*-periodate at neutral to slightly acidic pH values. Oxidized gp120 was combined with KLH under neutral to slightly basic conditions, and the resulting Schiff bases were reduced with either sodium cyanoborohydride or amine boranes, the latter being equally effective but less toxic reducing agents (Cabacungan *et al.*, 1982). Changes in periodate concentration, pH conditions, and reducing agent failed to yield CD4-reactive conjugates. Similarly, no significant CD4 binding activity was observed in gp120 self-conjugates prepared as above in the absence of KLH. The gp120 self-conjugates were analyzed in the EC_{50} assay described above which employs a polyclonal anti-gp120 coating antibody.

This activity loss could reflect intramolecular linkages between oxidized glycans and critical lysine residues on gp120 or oxidation of periodate-sensitive amino acids such as methionine (Geoghegan and Stroh, 1992).

In addition, no significant CD4-binding was observed for gp120-KLH conjugates prepared using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). EDC conjugation reactions were carried out in one- and two-step procedures in the presence and absence of sulfo-*N*-hydroxysuccinimide. The protocols targeted both primary amino and carboxyl groups on gp120. Although the conjugates lacked detectable CD4-binding activity, SDS-PAGE analysis of the conjugates indicated partial to complete coupling of gp120 to KLH (data not shown), suggesting that the procedures altered or masked the CD4-binding site on gp120.

SDS-PAGE analysis of gp120-KLH conjugates:

Conjugates possessing measurable CD4-binding activity were analyzed by SDS-PAGE to assess the extent of crosslinking. As expected, the crosslinked species were too large to enter the gel, but the degree of crosslinking could be estimated from the intensity of the unconjugated gp120 and KLH bands. As indicated in Figure 7, KLH was essentially completely crosslinked into high molecular weight species that are nearly completely excluded from the gel. Under the nondenaturing conditions of the conjugation procedures, KLH exists as a large multimeric protein, whose members would be expected to undergo intermolecular crosslinking. By comparing the intensities of the monomeric gp120 bands in the standard and conjugate lanes, we estimate that 50-90% of the gp120 was crosslinked in the conjugates prepared using SDBP and glutaraldehyde. As shown in Figure 7, increasing the glutaraldehyde concentration from 0.075% to 0.25% increased the degree of crosslinking, but only at the expense of reduced CD4-binding activity (*cf.* Figure 6). At this phase of the project, we do not plan to remove the unconjugated gp120 from these preparations. In all cases, conjugates will be compared with unmodified gp120 for immunogenicity in small animals. As our studies seek to determine if conjugation leads to heightened immune responses, the unconjugated gp120 will not affect the outcome. If favorable conjugates are obtained, purification procedures will be then be developed to remove unreacted gp120 and thereby presumably increase the potency of the conjugate preparation.

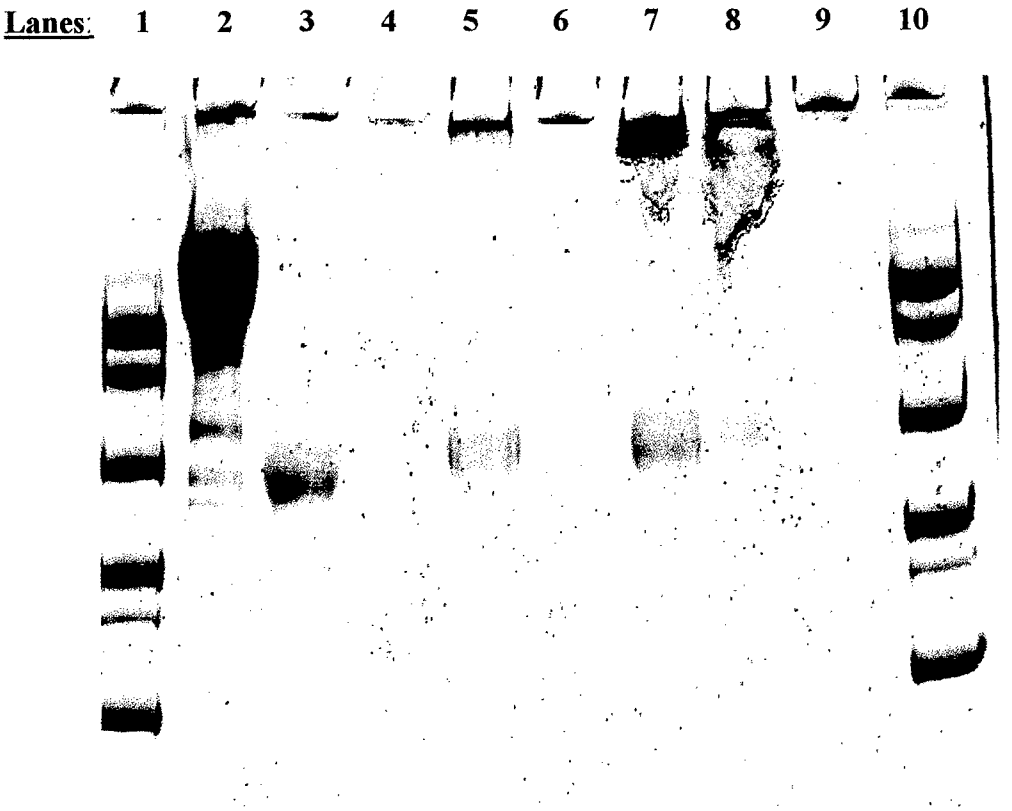

Figure 7. Scanned image of an SDS-PAGE gel containing HIV-1_{LAI} gp120-KLH conjugates. A 4-15% Ready-Gel (Bio-Rad Laboratories) was run under reducing conditions, stained with coomassie blue, and scanned on a laser densitometer (Molecular Dynamics).

Lanes 1 & 10: Pharmacia high molecular weight markers (212kd, 170kd, 116kd, 76kd, 53kd)
Lane 2: KLH standard, 10 μg
Lane 3: HIV-1_{LAI} gp120 standard, 10 μg
Lanes 4, 6 & 9: Empty
Lane 5: HIV-1_{LAI} gp120-KLH conjugate prepared with 3 mg/ml SDBP, 20 μg
Lane 7: HIV-1_{LAI} gp120-KLH conjugate prepared with 0.075% glutaraldehyde, 20 μg
Lane 8: HIV-1_{LAI} gp120-KLH conjugate prepared with 0.25% glutaraldehyde, 20 μg

The immunogenicity of the gp120-KLH conjugates is being assessed in rodents with future immunogenicity studies planned for the baboons.

CONCLUSIONS

During the first year of this contract, we have successfully expressed high levels of the recombinant envelope glycoprotein gp120 of HIV-1_{LAI} and HIV-1_{JR-FL} and developed a highly effective, nondenaturing purification method. The expressed gp120 recombinant proteins are highly pure as demonstrated by SDS-PAGE analysis. They were shown to retain their ability to bind to soluble CD4 in an ELISA format, and to cell surface-bound CD4 as determined by FACS analysis. Immunizations of guinea pigs with unconjugated gp120 derived from HIV-1_{LAI} or HIV-1_{JR-FL} in alum or in Ribi Detox adjuvants yielded relatively high serum anti-gp120 antibody titers which increased following sequential immunizations reaching a maximum of 1:250,000 to 1:500,000. Importantly, the gp120-induced immune responses also inhibited the binding of gp120 to CD4. Interestingly, the purified gp120 conjugated to tetanus toxoid elicited only a moderate anti-gp120 antibody response in BALB/C and C57BL mice. Because of this and the possibility of immunosuppression mediated by pre-existing antibodies to tetanus toxoid in humans, the purified gp120 was conjugated to KLH. SDBP- and glutaraldehyde-crosslinked gp120-KLH conjugates demonstrated good CD4-binding activity in a conjugate-specific ELISA. The extent of gp120 crosslinking in these conjugates was determined to be 50%-90% by SDS-PAGE. Immunogenicity studies of the gp120-KLH conjugates in rodents are in progress.

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